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Oxidants and Ca^{2+} induce PGC-1 α degradation through calpain

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Abstract

Peroxisome proliferator activator receptor γ coactivator 1 α (PGC-1 α) is a transcriptional coactivator known to mediate mitochondrial biogenesis. Whereas PGC-1 α transcription is regulated by a variety of signaling cascades, the mechanisms of PGC-1 α degradation have received less investigation. Thus, we investigated the mechanisms responsible for PGC-1 α degradation in renal proximal tubular cells (RPTC). Amino acid sequence analysis of the PGC-1 α protein revealed three PEST sequence-rich regions, predictive of proteolysis by calpains and/or the proteasome. Under basal conditions, treatment with the protein synthesis inhibitor cycloheximide resulted in rapid degradation of PGC-1 α ($t_{1/2}$ = 38 min), which was blocked by the proteasome inhibitor epoxomicin, but not the calpain inhibitor calpeptin. Oxidant exposure resulted in the degradation of both endogenous and adenovirally over-expressed PGC-1 α , which was inhibited by calpeptin but not epoxomicin. Thapsigargin-induced release of ER Ca^{2+} also stimulated calpain-dependent, epoxomicin-independent degradation of PGC-1 α . Finally, Ca^{2+} addition to lysates of RPTC over-expressing PGC-1 α resulted in calpeptin-sensitive, epoxomicin-insensitive degradation of PGC-1 α . In summary, we suggest two distinct mechanisms regulate PGC-1 α : basal PGC-1 α turnover by proteasome degradation and oxidant- and Ca^{2+} -mediated PGC-1 α degradation through calpain.

Keywords

PGC-1 α ; Calpain; Proteasome; Calcium; Oxidant; Kidney; Mitochondrial biogenesis

Because mitochondrial dysfunction is associated with a variety of disease states and acute organ injury, elucidating the mechanisms responsible for the regulation of mitochondrial function is essential for development of new therapeutic strategies [1–13]. Mitochondrial biogenesis ameliorates a variety of disease processes such as neurodegeneration and muscular dystrophy, and accelerates the recovery of mitochondrial and cellular functions in injured renal cells [12,14,15]. Strategies have been developed to screen for novel small molecule activators of the mitochondrial biogenesis regulator PGC-1 α [16]. Whereas these strategies have focused on identifying molecules that regulate the transcription of PGC-1 α , other groups have focused on targeting post-translational modifications known to increase the expression and activity of PGC-1 α [17–19]. Small molecule flavone, isoflavone, and stilbene structures have been identified as activators of SIRT1, a known deacetylase of PGC-1 α and an activator thereof [17–21].

Activation of p38 MAPK results in the phosphorylation of PGC-1 α at several critical residues within its repression domain, resulting in the dissociation of a myb160 binding protein and increases in the transcriptional activity of PGC-1 α [22]. Phosphorylation of PGC-1 α by p38 also increases the $t_{1/2}$ of PGC-1 α from approximately 2 to 6 h [23]. These data support the idea

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that modulating p38 MAPK activity and other mechanisms directed towards extending the $t_{1/2}$ of PGC-1 α may be a viable therapeutic option to promote mitochondrial biogenesis and treat diseases associated with mitochondrial dysfunction.

Several groups have shown that PGC-1 α is rapidly turned over by a proteasomal dependent pathway and during periods of oxidative stress, PGC-1 α translocates to the nucleus in an SIRT1-dependent manner to stimulate a compensatory increase in mitochondrial biogenesis prior to proteasomal degradation [24]. In these studies the total pool of PGC-1 α protein was not altered as the depletion of PGC-1 α was offset by the increased transcription of PGC-1 α .

Sano et al. identified several regions of PGC-1 α that are enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST sequences) [25]. These sequences are known target sequences for a variety of cysteine proteases, including the Ca^{2+} -activated cysteine protease calpain [26–33]. Thus, we hypothesized that PGC-1 α may also be degraded by a calpain during conditions of Ca^{2+} overload. The goal of this study was to examine the various mechanisms mediating degradation of PGC-1 α in renal cells under basal and pathologic conditions.

Materials and methods

Reagents

Anti-GAPDH and anti-PGC-1 α (H300) antibodies were purchased from Fitzgerald Antibodies (Concord, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. A Myc antibody was obtained from Cell Signaling (Danvers, MA). The calpain substrate, suc-Leu-Leu-Val-Tyr-AMC (sLLVY-AMC), was purchased from Bachem (King of Prussia, PA). Calpeptin and purified calpain I from porcine kidney were obtained from EMD Biosciences (La Jolla, CA). All secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL). Anti-PGC-1 α was used at 1:300, whereas all other antibodies were used at 1:1,000. All other chemicals were purchased from Sigma (St. Louis, MO).

Isolation and culture of renal proximal tubules

Female New Zealand White rabbits (2 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved conditions as previously described [34]. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 $\mu\text{g}/\text{ml}$), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium. Confluent RPTC were used for all experiments. RPTC monolayers were treated with various compounds or diluent (DMSO) for 24 h.

Calpain 1 activity

Calpain 1 activity was determined by a fluorometric assay using purified calpain 1 and the calpain substrate sLLVY-AMC as previously described [35]. Briefly, calpain 1 (final concentration 0.76 $\mu\text{g}/\text{ml}$) was added to assay buffer containing various concentrations of calpain inhibitors and Ca^{2+} (final concentrations: 20 mM HEPES, 150 mM KCl, 5 mM DTT, 6 mM Ca^{2+} , pH 7.4). After a brief period of mixing, sLLVY-AMC (final concentration 50 μM) was added. Activity was measured under linear conditions as a function of AMC hydrolysis using an excitation wavelength of 355 nm and an emission wavelength of 444 nm.

Preparation of cell lysates and immunoblot analysis

RPTC were washed twice with PBS without Ca^{2+} and Mg^{2+} and harvested in cell lysis buffer from BioVision (Mountain View, CA). Immunoprecipitations were performed by incubating lysates (1 mg cellular protein) overnight with Protein G coated agarose beads to preclear non-specific proteins. Lysates were incubated with antibody for 12 h, followed by a 3 h incubation with protein G coated agarose beads, and then centrifugation. Samples were subsequently boiled and prepared for electrophoresis. After protein disruption by sonication for 30 s, equal amounts of protein lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. After treatment with 5% skim milk or BSA at 4 °C overnight, membranes were incubated with various antibodies for 2 h and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Bound antibodies were visualized by chemiluminescence detection on an Alpha Inotech imaging system (San Leandro, CA).

Replication-deficient adenovirus infection

Recombinant adenovirus expressing PGC-1 α or a nuclear GFP control were a generous gift from Bruce Spiegelman (Harvard Medical School) [36]. Viral titers were determined by plaque formation assay using HEK293 cells and expressed as plaque-forming units (pfu)¹. RPTC were infected with each virus at a multiplicity of infection (MOI) of 30 for 24 h at 37 °C in a humidified, 5% CO₂ incubator. Afterward, the cultures were placed in normal culture media for an additional 24 h, at which point all measurements were taken. At an MOI of 30, 99% of the cells showed expression of the viral gene insert as indicated by flow cytometry (data not shown).

Statistical analysis

Data are presented as means $\pm$ SE and were subjected to one- or two-way ANOVA as appropriate. Multiple means were compared using the Student–Newman–Keuls test, with $p < 0.05$ considered statistically significant. RPTC isolated from an individual rabbit represent a single experiment ($n = 1$). Experiments were performed with 2–5 plates of cells and repeated until an n of at least 3 was reached.

Results

We observed that the basal level of PGC-1 α in RPTC was low. To characterize the degradation of PGC-1 α in RPTC, PGC-1 α was over-expressed using an adenoviral vector and degradation was evaluated in the presence of the protein synthesis inhibitor cycloheximide (100 μM). Adenovirally over-expressed PGC-1 α was degraded with a calculated $t_{1/2}$ of ~ 38 min (Fig. 1), suggesting that PGC-1 α has a rapid turnover rate in RPTC under control conditions.

We and others [25] determined that PGC-1 α has numerous PEST sequences (Fig. 2). Because cysteine proteases are known to cleave at PEST sequences, we examined several cysteine protease inhibitors for their ability to block PGC-1 α degradation. MG-132 (10 μM), a prototypical proteasome inhibitor, inhibited the activity of purified calpain 1, consistent with reports of MG-132 cross reacting with a variety of cysteine proteases (Fig. 3) [37]. The calpain-specific inhibitor, calpeptin (100 μM), also inhibited calpain 1 activity. In contrast, the more specific proteasome inhibitor, epoxomicin (10 μM) [38–40], had no effect on calpain 1 activity.

RPTC over-expressing PGC-1 α were treated with cycloheximide to block PGC-1 α synthesis, permitting the observation of PGC-1 α degradation in isolation. Prior to the addition of cycloheximide, RPTC were pretreated with DMSO, MG-132 (10 μM), epoxomicin (10 μM),

¹Abbreviations used: RPTC, renal proximal tubular cells; pfu, plaque-forming units; MOI, multiplicity of infection; TBHP, *tert*-butyl hydroperoxide; P, proline; S, serine; T, threonine.

or calpeptin (100 μ M). Approximately 55% of PGC-1 α protein was degraded within the first hr with total degradation occurring 2 h after the addition of cycloheximide (Fig. 4). This degradation was inhibited by pretreatment with the proteasome/calpain inhibitor MG-132. Similarly, epoxomicin blocked PGC-1 α degradation at 1 h and approximately 80% at 2 h. In contrast, calpeptin had no effect on PGC-1 α at 1 h and blocked approximately 20% at 2 h. In a complimentary experiment, MG-132, epoxomicin, or calpeptin were added to RPTC in which PGC-1 α had been over-expressed in the absence of cycloheximide. Adenoviral-induced PGC-1 α accumulated within 1 h of treatment with both MG-132 and epoxomicin, but did not accumulate in the presence of calpeptin (Fig. 5). We suggest that basal degradation of PGC-1 α is mediated by the proteasome and not through calpains.

Because proteasomal degradation pathways can be artifacts in over-expression systems, MG-132, epoxomicin, or calpeptin were added to RPTC. Addition of MG-132 or epoxomicin to RPTC resulted in the accumulation of endogenous PGC-1 α by 4 h and remained increased at 8 h (Fig. 5). In contrast, calpeptin had no effect on PGC-1 α accumulation at any time point. Hence, PGC-1 α degradation in RPTC or in RPTC over-expressing PGC-1 α is mediated by the proteasome but not by calpain.

Because oxidants have been reported to increase Ca^{2+} and calpain activity [41–43], the mechanism of oxidant-induced PGC-1 α degradation was evaluated. PGC-1 α was immunoprecipitated from RPTC treated with either DMSO or *tert*-butyl hydroperoxide (TBHP, 200 μ M for 45 min) and subjected to immunoblot analysis. TBHP exposure promoted the degradation of PGC-1 α (Fig. 6). Because oxidants have been reported to induce a compensatory increase in PGC-1 α expression [13,14,24], the next experiment was conducted in the presence of cycloheximide. RPTC over-expressing PGC-1 α were pretreated with epoxomicin and exposed to TBHP, which induced degradation of PGC-1 α that was inhibited by calpeptin (Fig. 6). Calpeptin in the absence of TBHP had no effect on PGC-1 α . These data reveal that oxidant stress promotes the degradation of PGC-1 α in a calpain-dependent manner.

To further explore the role of Ca^{2+} and calpain in PGC-1 α degradation, RPTC were pretreated with epoxomicin to eliminate proteasomal degradation and cycloheximide to eliminate PGC-1 α synthesis. RPTC were then treated with thapsigargin (4 μ M) [44] to release ER Ca^{2+} and PGC-1 α expression was measured 1 h later. Release of ER Ca^{2+} promoted the degradation of PGC-1 α and this degradation was blocked by calpeptin (Fig. 7). The calpain inhibitor PD150606 (300 μ M), a non-peptide inhibitor of “typical” calpains also blocked PGC-1 α degradation produced by thapsigargin. We suggest that the release of ER Ca^{2+} promotes calpain-dependent degradation of PGC-1 α .

We also examined the degradation of PGC-1 α in RPTC lysates. RPTC over-expressing PGC-1 α were lysed and incubated in a calpain activity buffer for 5 min. Ca^{2+} promoted the degradation of PGC-1 α and this degradation was blocked by pretreatment with calpeptin, but not with epoxomicin (Fig. 7), providing further evidence that Ca^{2+} -induced degradation of PGC-1 α is mediated by a calpain.

Finally, we determined whether purified calpain 1 was capable of degrading PGC-1 α . Myc-PGC-1 α was immunoprecipitated in its native form on protein A-coated agarose beads using an anti-myc antibody and exposed to purified calpain 1 in a calpain activity buffer for 5 min. Calpain 1 promoted the degradation of PGC-1 α (Fig. 8).

Discussion

Because the regulation of mitochondrial biogenesis, degradation, and function is critical to the health and survival of cells, understanding the mechanisms by which regulators of

mitochondrial biogenesis such as PGC-1 α are regulated is critical to develop interventions that can influence mitochondrial biology and function.

In this study, we demonstrate that primary cultures of RPTC rely on the proteasomal degradation pathway to maintain basal levels of PGC-1 α . These results are consistent with several reports demonstrating that the proteasome is a key mediator of PGC-1 α under basal conditions [24,25,45]. Additionally, it is important to note that the calpain inhibitor calpeptin, did not affect PGC-1 α stability under conditions of proteasome inhibition, suggesting that calpain does not play a significant role in the maintenance of PGC-1 α expression under basal conditions.

Interestingly, adenovirally over-expressed PGC-1 α is rapidly degraded in primary cultures of RPTC with a $t_{1/2}$ of ~38 min (Fig. 3). This $t_{1/2}$ is much shorter than that observed in immortalized renal cell lines in the past (apparent $t_{1/2} > 1$ h) [25]. Another group has reported the $t_{1/2}$ of PGC-1 α to be ~2.3 h in BOSC cells [46], which suggests that the rates of PGC-1 α degradation may vary significantly depending on the cell type. This is the first report characterizing the basal turnover of PGC-1 α in a primary cell culture model whose high level of aerobic metabolism and low level of glycolysis is similar to that found *in vivo*, which may more accurately represent physiological conditions.

Exposure of RPTC to a toxic concentration of the oxidant TBHP promoted the degradation of both endogenous and adenovirally-expressed PGC-1 α . Oxidant exposure this severe is sufficient to induce ~50% cell death over 24 h, the remaining sublethally injured cells have significant mitochondrial dysfunction [13,47]. PGC-1 α degradation in oxidant-exposed RPTC was blocked by pretreatment with calpeptin, suggesting that the oxidant-induced degradation of PGC-1 α is calpain-dependent. The degradation of PGC-1 α by a proteasome-independent mechanism is novel.

Because oxidants are known to promote the release of ER Ca²⁺ and activate cytosolic calpains [41–43], we determined whether ER Ca²⁺ release and subsequent calpain activation resulted in the degradation of PGC-1 α . Under basal conditions, the release of ER Ca²⁺ resulted in PGC-1 α degradation that was calpain-dependent and proteasome-independent. Incubating RPTC lysates in a Ca²⁺-containing buffer promoted calpain-dependent and proteasome-independent degradation of PGC-1 α . Furthermore, immunoprecipitated PGC-1 α was degraded by purified calpain 1. Taken together, these results provide strong evidence that ER Ca²⁺ release and calpain are key mediators of PGC-1 α degradation.

“Typical” calpain family members, (calpains 1, 2, 3, 4, 8, 9, 11, 12, and 13) contain a Ca²⁺ binding EF hand domain, absent in atypical calpains, that is critical for activation and enzymatic catalysis [48]. The calpain inhibitor PD150606 selectively binds to the EF hand domain of typical calpains to block activation, thus providing a chemical tool to distinguish between the activity of typical and atypical calpains [49,50]. Pretreatment of RPTC with PD150606 blocked thapsigargin-induced degradation of PGC-1 α , suggesting that PGC-1 α is degraded by a typical calpain during periods of Ca²⁺ overload.

The recognition of calpain substrates is known to be regulated in part by post-translational modification [51]. Because both the activity and expression of PGC-1 α are tightly regulated by phosphorylation and acetylation, it is likely that post-translational modification of PGC-1 α may contribute to its instability during periods of calcium overload or oxidative stress [52]. Additionally, typical calpain family members show limited activity under conditions of low Ca²⁺ and are activated during oxidative stress, this provides a more likely mechanism by which calpains may cleave PGC-1 α , limit the up-regulation of PGC-1 α and contribute to the cell death process after oxidant injury. In cells sublethally injured, the up-regulation of

PGC-1 α expression may be maintained, presumably by the lack of calpain activation, promoting cell-survival and may promote the recovery of mitochondrial function as reported previously [13]. This novel mechanism of PGC-1 α degradation may provide a new pathway by which cells signal cell death during the early stages of the injury process and then allow for the subsequent recovery of mitochondrial function in surviving sub-lethally injured cells. Increases in PGC-1 α in response to mild oxidative stress in other models suggest that this protein may be a key mediator of the cell-survival response.

In conclusion, we have identified two distinct pathways which mediate the degradation of PGC-1 α . Under basal conditions, PGC-1 α is rapidly degraded via the ubiquitin-proteasome system, whereas PGC-1 α is degraded via a calpain-dependent mechanism during severe oxidative stress and cell injury and death. We propose that PGC-1 α is increased following cell injury to promote cell-survival and ultimately to increase or replace mitochondria. If the injury is severe enough to increase cytosolic Ca²⁺, calpain activation ensues to degrade PGC-1 α , removing a cell-survival signal, and to contribute cell death.

Acknowledgments

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Fig. 1.

PGC-1α is rapidly degraded in primary cultures of RPTC. PGC-1α was over-expressed using an adenovirus in RPTC for 48 h. RPTC were then exposed to the protein synthesis inhibitor cycloheximide (100 μM), PGC-1α levels were measured at various time points and densitometry was performed to determine the $t_{1/2}$ of PGC-1α in this system ($t_{1/2} = \sim 38$ min).

AA#

0 MAWDMC SQDSVW SDIECAALVGEDQPLCPDLPELDL SELDVNOLD TDSFL
 GGLKWCS DQSEHSNQYNNEPANIFE KIDEENEANLLAVLTETLDSLPVDED

100 GLPSFDALTDGAVTTDNEASPS S MPDGT PPPQEAEEPSLLKLLAPANTQ
 LSYNEC SGLSTQNHAANH THIR TNPAIVK TENSWSNKAK SICQQQKPQRR

200 PCSELLKYLT TNDDPPHTKPTENRNS SRDKCASKKK SHTQPQ SQHAQA KP
TTSLPLTPESPNDPKGSPFENK TIER TL SVEL SG TAGL TPP TTPPHKANQDN

300 PFKASPKLKPSCK TVVPPPTKRARY SEC SG TOG SH SKKGPEQ SELYAQLS
 KSSGLSRGHEERK TKRPSLR LFGDHDYCQSLN SKTDILINISQELQDSRQLD

400 FKDA SCDWQGHIC SSTD SGQCYL RETLEASKQVSPCSTRKQLQDQEIRAE
 LNKHFGHPCQAVFDDKSDK TSEL RDGDF SNEQFSKLPVFINSGLAMDGLF

500 DDSEDE SDKLSYPWDG TQPYSLFDVSPSCS SFNSPCRD SVSPPKSLF SQR
 PQRMRSRSRSF SRHRSCSRSPY SRSR SRSPG SRSSSR SCYYYESSHYRHR

600 THRNSPLYVRSR SRSPY SRRPRYDSYEAY EHERLKRDEYRKEHEKRE SER
 AKQRE RQKQKAIEERRVIYVGKIRPD TTR TELRDRFEVFG EIEECTVNLRDD

700 GDSYG FITYRYTCD AFAALENGYTLRRSNETDFELYFCGRKQFF KSNYADL
DTNSDDFDPA STKSKYDSLDFD SLLKEAQRSLRR

Fig. 2.

PGC-1 α contains several PEST domains indicative of cysteine protease cleavage. The amino acid sequence of PGC-1 α contains three regions rich in proline, glutamic acid, serine, and threonine (AA 77–139, 248–264, and 744–762).

Fig. 3.

Epoxomicin is a specific inhibitor of the proteasome. Cleavage of the calpain substrate sLLVY-AMC was used to monitor calpain 1 activity in the presence or absence of the cysteine protease inhibitors MG-132 (10 μ M), calpeptin (100 μ M), and epoxomicin (10 μ M). Data are presented as means $\pm$ SE, $n = 3$ for each group. Different letters represent means that are different from each other ($p < 0.05$).

Fig. 4. Proteasomal inhibition blocks basal degradation of PGC-1α. PGC-1α was over-expressed in RPTC for 48 h. RPTC were then treated with cycloheximide in the presence or absence of various inhibitors of the proteasome (MG-132 and epoxomicin) or calpain (MG-132 and calpeptin) and protein degradation was measured by immunoblot at 1 and 2 h time points. Densitometry was performed to quantify the degradation at each time point. Different letters represent means that are different from each other ($p < 0.05$).

Fig. 5. Inhibition of the proteasome promotes the accumulation of PGC-1 α . RPTC were treated with either the proteasome/calpain inhibitor MG-132, the proteasome inhibitor epoxomicin, or the calpain inhibitor calpeptin for 1 h in cells over-expressing PGC-1 α or at 0, 2, 4, and 8 h in cells in which PGC-1 α had not been over-expressed. PGC-1 α expression was then measured by immunoblot. Blots are representative of at least three separate experiments.

Fig. 6.

Oxidants promote the calpain-mediated degradation of PGC-1 α . RPTC were exposed to TBHP (200 μ M for 45 min), PGC-1 α was immunoprecipitated and PGC-1 α levels determined by immunoblot analysis (a). RPTC over-expressing PGC-1 α were pretreated with epoxomicin (10 μ M) and cycloheximide (100 μ M) and then exposed to TBHP (200 μ M for 45 min) in the presence and absence of calpeptin (100 μ M). PGC-1 α levels were determined by immunoblot analysis (b). Blots are representative of at least three experiments.

Fig. 7. ER Ca^{2+} release and a typical calpain mediates PGC-1 α degradation. RPTC over-expressing PGC-1 α were pretreated with epoxomicin (10 μM) and cycloheximide (100 μM) then exposed to thapsigargin (4 μM for 1 h) to promote the release of ER Ca^{2+} in the presence or absence of calpeptin or PD150606 (300 μM) (a). PGC-1 α levels were determined by immunoblot analysis. RPTC over-expressing PGC-1 α were lysed in either an EGTA-buffered solution or Ca^{2+} -containing buffer in the presence and absence of calpeptin (100 μM) or epoxomicin (10 μM) for 5 min and PGC-1 α levels were determined by immunoblot analysis (b). Blots are representative of at least three experiments.

Fig. 8. Calpain 1 cleaves native PGC-1 α . Adenoviral over-expression of PGC-1 α with a C-terminal myc tag was immunoprecipitated on protein A-coated agarose beads and incubated with purified calpain 1 (5 min). PGC-1 α levels were determined by immunoblot analysis. Blots are representative of at least three experiments.